Galdes, A., Auld, D. S., & Vallee, B. L. (1983) *Biochemistry 22*, 1888–1893.
Hangauer, D. G., Mozingo, A. F., & Matthews, B. W. (1984) *Biochemistry 23*, 5730–5741.
Hill, H. A. O., Sammes, P. G., & Waley, S. G. (1980) *Philos. Trans. R. Soc. London, B 289*, 333–344.
Holmquist, B., Kaden, T. A., & Vallee, B. L. (1975) *Biochemistry 14*, 1454–1461.
Kannan, K. K., Petef, M., Fridborg, K., Cid-Dresdner, H., & Lorgren, S. (1977) *FEBS Lett. 73*, 115–119.
Knott-Hunziker, V., Petursson, S., Waley, S. G., Jaurin, B., & Grundstrom, T. (1982) *Biochem. J. 207*, 315–322.
Lindskog, S. (1966) *Biochemistry 5*, 2641–2646.
Little, C., Emanuel, E., Gagnon, J., & Waley, S. G. (1985) *Biochem. J.* (in press).
Maurel, P. (1978) *J. Biol. Chem. 253*, 1677–1683.
Meloche, H. P., & Glusker, J. P. (1973) *Science (Washington, D.C.) 181*, 350–352.
Mozingo, A. F., & Matthews, B. W. (1984) *Biochemistry 23*, 5724–5729.
O'Callaghan, C. H., Morris, A., Kirby, S. M., & Shingler, A. H. (1972) *Antimicrob. Agents Chemother. 1*, 283–288.
Page, M. I. (1984) *Acc. Chem. Res. 17*, 144–151.
Pratt, R. F., & Govardhan, C. P. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1302–1306.
Privalov, P. L. (1979) *Adv. Protein Chem. 33*, 167–241.
Rodiguin, N. M., & Rodiguina, E. N. (1964) *Consective Chemical Reactions*, pp 111–131, Van Nostrand, Princeton, NJ.
Sabath, L. D., & Abraham, E. P. (1966) *Biochem. J. 98*, 11c–13c.
Saino, Y., Kobayashi, F., Inoue, M., & Mitsuhashi, S. (1982) *Antimicrob. Agents Chemother. 22*, 564–570.
Selwyn, M. J. (1965) *Biochim. Biophys. Acta 105*, 193–195.
Tan, H., & Lovrien, R. (1972) *J. Biol. Chem. 247*, 3278–3285.
Taylor, P. W., King, R. W., & Burgen, A. S. V. (1970) *Biochemistry 9*, 3894–3902.
Vallee, B. L., & Galdes, A. (1984) *Adv. Enzymol. Relat. Areas Mol. Biol. 56*, 283–430.
Wharton, C. W., & Szawelski, R. J. (1982) *Biochem. J. 203*, 351–360.

NMR Studies of Chromomycin A_3 Interaction with DNA†

Elisha Berman,*,‡ Stephen C. Brown,§ Thomas L. James,§ and Richard H. Shafer*,§

Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143, and Department of Organic Chemistry, The Weizmann Institute of Science, 76100 Rehovot, Israel

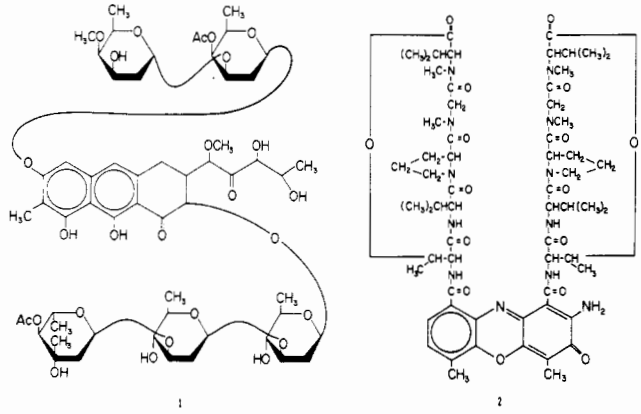
Received February 14, 1985

ABSTRACT: The binding of chromomycin A_3 to calf thymus DNA and poly(dG-dC) has been studied by ^{13}C and ^{1}H NMR with emphasis on the mode of binding, the role of Mg^{2+}, and pH effects. The most prominent changes in the DNA base pair ^{13}C NMR resonances upon complexation with chromomycin were observed for G and C bases, consistent with the G-C preference exhibited by this compound. Comparison of the ^{13}C spectrum of DNA-bound chromomycin A_3 with that of DNA-bound actinomycin D, a known intercalator, showed many similarities in the base pair resonances. This suggested the possibility that chromomycin A_3 binds via an intercalative mechanism. ^{1}H NMR studies in the imino proton, low-field region of the spectrum provided additional evidence in support of this binding mode. In the low-field spectrum of chromomycin A_3 bound to calf thymus DNA, a small shoulder was observed on the upfield side of the G-C imino proton peak. Similarly, in the chromomycin A_3 complex with poly(dG-dC), a well-resolved peak was found upfield from the G-C imino proton peak. These results are expected for ligands that bind by intercalation. Futhermore, in both the calf thymus and poly(dG-dC) drug complexes (in the presence of Mg^{2+}) a broad peak was also present downfield ($\sim$16 ppm from TSP) from the DNA imino protons. This was attributed to the C-9 phenolic hydroxyl proton on the chromomycin chromophore. Visible absorbance spectra at different pH values showed that the role of Mg^{2+} in the binding of chromomycin A_3 to DNA is more than simple neutralization of the drug's anionic change.

Chromomycin A_3, an antitumor antibiotic that belongs to the aureolic acid group (Remers, 1979), is composed of the aglycon chromomycinone and five sugar units consisting of chromoses A, B, C, and D (Miyamoto et al., 1967). The complete structure of chromomycin A_3 (structure **1**) was established only recently by ^{1}H and ^{13}C NMR spectroscopy (Thiem & Meyer, 1979). It is of importance to note that the chromophore has a pK_a of $\sim$7.0 and that divalent cations, in particular Mg^{2+}, are needed for the strong interaction of the drug with DNA to take place (Hayasaka & Inou, 1969; Nayak et al., 1973). It was also found that chromomycin A_3 shows a preference of G-C base pairs in DNA (Behr et al., 1969; Berman & Shafer, 1983; Van Dyke & Dervan, 1983) and that the sugar side chains play a role in the binding to DNA (Behr et al., 1969; Berman & Shafer, 1983). Similar properties were observed for related members of the aureolic acid group such

† This work was supported by U.S. Public Health Service Grant CA 27343 awarded by the National Institutes of Health, DHHS (R.H.S.), and a grant from the Cancer Research Coordinating Committee of the University of California (E.B.).
‡ The Weizmann Institute of Science.
§ University of California.

0006-2960/85/0424-6887$01.50/0



as mithramycin and olivomycin (Thiem & Meyer, 1981; Van Dyke & Dervan, 1983). Recently it was found that the interaction of chromomycin A_3 with DNA shows some similar features to the interaction of actinomycin D (structure **2**) with DNA (Berman & Shafer, 1983). However, while actinomycin D, which has also been studied extensively by NMR methods (Patel, 1974a–c; Krugh & Chen, 1975; Krugh & Chen Chiao, 1977; Brown et al., 1982, 1984), intercalates between DNA base pairs, there is no conclusive data about the mode of binding of chromomycin A_3 to DNA (Waring, 1970; Remers, 1979).

In the present study we have used NMR methods to investigate the nature of the interaction of chromomycin A_3 with DNA with respect to the question of intercalation, the role of Mg^{2+} cations, and the importance of the sugar side chains in the drug–DNA complex. We have studied aqueous solutions of chromomycin A_3 with respect to self-aggregation (Hayasaka & Inoue, 1969) as well as the effect of pH and temperature on its binding to DNA fragments. We have also compared our results for chromomycin–DNA interactions with those observed for the G-C-specific intercalator actinomycin D and for the nonintercalating A-T-specific bisbenzimide Hoechst 33258.

Materials and Methods

Calf thymus DNA (type I), chromomycin A_3, actinomycin D, and Hoechst 33258 were obtained from Sigma Chemical Co., St. Louis, MO. Poly(dG-dC) was obtained from P-L Biochemicals, Milwaukee, WI. Deoxyribonuclease II was purchased from Millipore Co., Freedom, NJ. All other chemicals used were reagent grade quality.

Preparation of DNA Samples. Two grams of calf thymus DNA was dissolved in 350 mL of 0.1 M sodium acetate buffer containing 2 mM zinc chloride at pH 4.9. To the stirred solution, at room temperature was added 11 000 units of deoxyribonuclease II enzyme. An additional 11 000 units was added after 3 h of incubation. The incubation was terminated after 6 h by extraction (×5) with 5:1 chloroform/isoamyl alcohol. The DNA fragments were precipitated from 70% ethanol solution. Pellets were dried under vacuum. The DNA was redissolved in 20 mM tris(hydroxymethyl)aminomethane (Tris) buffer at pH 8.0, centrifuged at 7000 rpm for 1 h, and dialyzed (×4, volume ratio 1/50). The yield was estimated to be ~70%. The average DNA length was determined by slab polyacrylamide gel (15%) electrophoresis under native conditions and was found to be equivalent to 70 ± 30 base pairs by using *Hae*III fragments of ϕX174-RF DNA. Thermal denaturation curves were determined on a Gilford 2600 spectrophotometer. The T_m was found to be 55 °C and accompanied by a change in the 260-nm absorbance over the temperature range of 20–80 °C of ~20%. Poly(dG-dC) samples were sonicated to a length of 200 ± 100 base pairs for ^{1}H NMR studies. DNA concentrations were determined spectroscopically on the basis of the molar (nucleotide) extinction coefficients of ϵ_{260} = 6600 for calf thymus DNA and ϵ_{254} = 7100 for poly(dG-dC).

Visible Absorbance Studies. For spectrophotometric studies of chromomycin A_3 binding to DNA (see Figure 4), the same stock solutions of DNA, chromomycin, and magnesium chloride in the appropriate buffer (20 mM Tris) were used to generate the required solutions. The pH was adjusted with either sodium hydroxide or hydrochloric acid. All determinations were made with the appropriate reference solutions containing the same ingredients but without chromomycin. The solutions were refrigerated overnight and allowed to reach room temperature (22 °C, 4 h) before spectroscopic measurements were taken on a Cary 118 spectrophotometer.

NMR Samples. The digested DNA sample was concentrated down to 20 mL, in 20 mM Tris buffer, pH ~8.0, and 20 mM magnesium chloride, containing ~10% D_2O. The sample was divided into two equal volumes. To one part, chromomycin was added, and the solution was allowed to stand at room temperature for 4 h before running the ^{13}C NMR spectrum. Then a change in pH was carried out (for details, see Figure 3). The NMR spectrum of the other half-sample was recorded as a reference spectrum (Figure 2A) before adding actinomycin D to it (Figure 2C). The drugs were removed by ethyl acetate extraction (×6) for actinomycin D and chloroform (×12) for chromomycin A_3. Final extractions were effected with phenol (×3) and then ether (×3), and the samples were dialyzed against fresh buffer. These samples were then used again to obtain the ^{13}C NMR spectra of the actinomycin–DNA complex without magnesium chloride and the Hoechst 33258–DNA sample. Reference NMR spectra were recorded immediately before the drugs were added to the solutions. The exact final concentrations of the samples above and their respective pH values and temperatures in which the NMR experiments were run are noted in the figure captions.

NMR Studies. The ^{13}C spectra were obtained on a home-built spectrometer operating at 60.46 MHz for ^{13}C. Flat-bottom NMR tubes (20 mm o.d.) were used, and the sample length was 3.6–3.8 cm. The temperature of the samples was recorded by using a thermocouple at three positions along the length of the sample. Typically a temperature gradient of 3–4 °C was observed for the sample run at ~40 °C. Slightly larger (~8 °C) temperature gradients were observed for the aqueous solutions of chromomycin. The data were collected on a 1180 Nicolet computer by using 16K data points, a spectral window of 13158 Hz, and a 1-s recycle time. Line broadening of 10 Hz was used in all the DNA-containing samples. For other spectra the line broadening is noted in the figure captions.

^{1}H NMR spectra were obtained on the same spectrometer operating at 240 MHz on samples containing 10% D_2O at 15 °C. A jump and return sequence (Plateau & Gueron, 1982) was used to suppress the solvent signal. Chemical shifts are reported relative to internal TSP. See figure legends for additional details.

Results and Discussion

^{13}C NMR Studies

Chromomycin A_3 Alone. In aqueous solutions, chromomycin A_3 tends to self-aggregate with average clusters of approximately five monomers (Hayasaka & Inoue, 1969), while in organic solvents it is found in the monomeric state

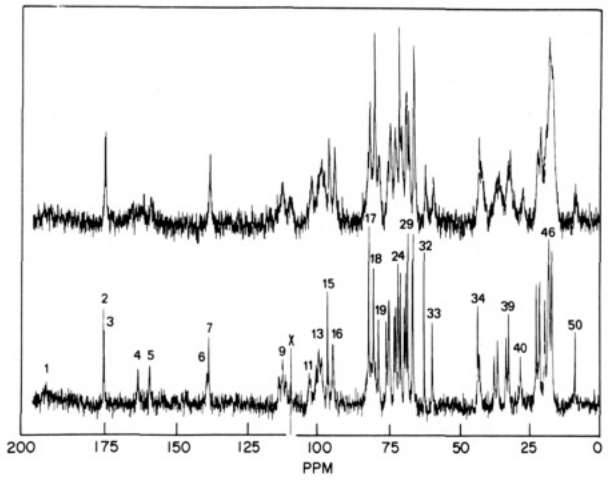


FIGURE 1: 60.46-MHz ^{13}C spectra of 8 mM chromomycin A_3 in 50 mM phosphate buffer (15% D_2O) at pH 8.3, 25 °C, 45 000 scans, and 3-Hz line broadening (bottom spectrum) and at pH 7.3, 20 °C, 56 000 scans, and 3-Hz line broadening (top spectrum).

(Thiem & Meyer, 1979). Figure 1 shows the effect of aggregation in the ^{13}C NMR spectra of chromomycin at different pH values. It displays the ^{13}C NMR spectrum of chromomycin at two different pH values, one at pH 7.3, near the pK_a (~7.0) value for chromomycin (Nayak et al., 1973), and the other at pH 8.3. As seen from Figure 1 there is a very strong self-aggregation at the lower pH value as manifested by the observed line widths. Clearly, the introduction of negative charge on the drug's chromophore eliminates most of the aggregation as shown in the ^{13}C spectrum obtained at pH 8.3. When the spectra shown in Figure 1 are compared, it is easily observed that some selected resonances remain relatively narrow. Since direct comparison between the ^{13}C spectra obtained in aqueous solution to those obtained in organic solvent (Thiem & Meyer, 1979) is not possible, we could not establish unequivocally the corresponding assignments for the resonances. The use of solvent mixtures such as water/methanol (Berman & Shafer, 1983) did not serve to simplify the assignment problem. Those resonances that retain their narrow line widths at pH 7.3 (peaks 18, 24, and 31) most likely correspond to either of the two terminal sugar residues or the chromophore side chain (Thiem & Meyer, 1979). These parts of the molecule are most likely to retain their relative flexibility in aqueous solution with the assumption that the aromatic part of the molecule is responsible for the observed aggregation. Most noticeable is the fact that only one of the five anomeric resonances has considerably sharpened up at pH 8.3 (peak 15). The two methoxy resonances (peaks 32 and 33) have also sharpened up at pH 8.3. One of the methoxy groups is found on a terminal sugar residue and the other on the side chain of the chromophore. Thus, it seems that the terminal sugar residue that is found on the disaccharide branch of the molecule (structure **1**) is the first sugar residue to gain motional mobility upon elevation of the pH from 7.3 to 8.3. Considerable improvement in the observed line width of the spectrum could only be effected further by adding small amounts of methanol to the aqueous solution (Berman & Shafer, 1983); even then, some residual aggregation may be present. The chemical shifts of the peaks observed in ths aqueous ^{13}C NMR spectrum of chromomycin at pH 8.3 are listed in Table I. The observed chemical shifts depend on the pH and the degree of aggregation, but most of these changes are not larger than 0.5 ppm.

Chromomycin–DNA Binding. (1) Analysis of the Spectrum. Upon mixing chromomycin A_3 with the DNA fragments, we expected to observe some spectral changes when the resultant ^{13}C NMR spectrum was compared with the initial ^{13}C NMR spectra of the drug and of DNA alone. In Figure 2 we compare the ^{13}C spectrum of DNA alone (Figure 2A) and in the presence of chromomycin (Figure 2B). Clearly in the aromatic region of the bases we see noticeable intensity

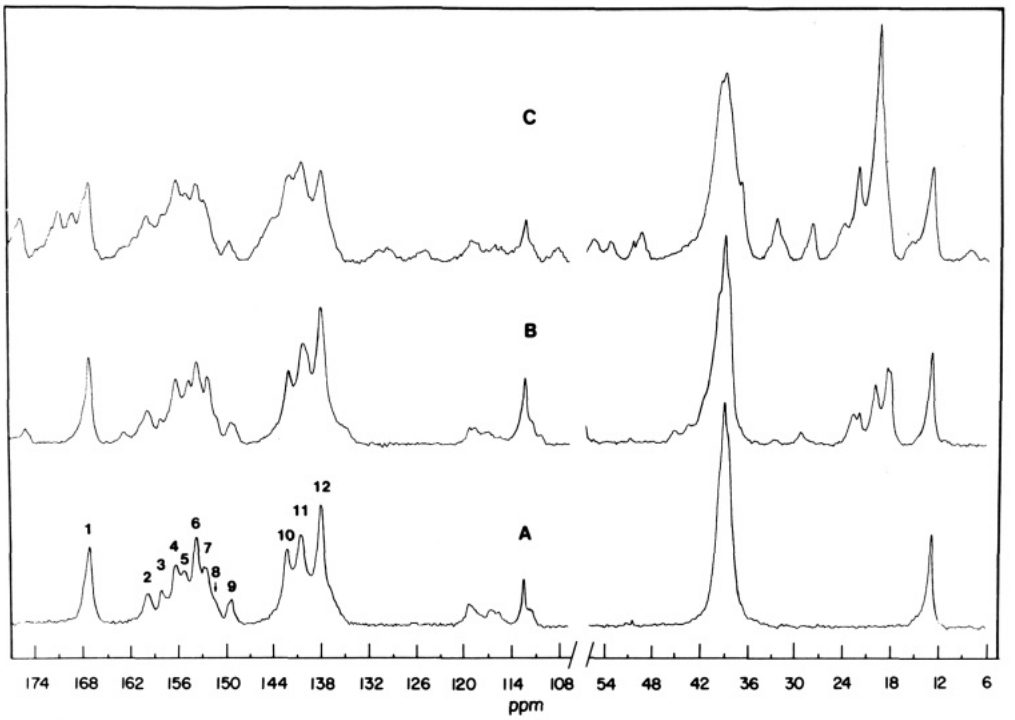


FIGURE 2: 60.46-MHz ^{13}C NMR spectra, displaying the aromatic region (178–107 ppm) and upfield region (56–6 ppm) of (A) native DNA, 137 000 scans, (B) DNA and 8 mM chromomycin A_3, 165 000 scans, and (C) DNA and 8 mM actinomycin D, 136 000 scans. Other conditions: 150 mM DNA (nucleotides) in aqueous solution (15% D_2O) containing 20 mM Tris and 20 mM $MgCl_2$, pH 8.0, T = 38–42 °C. The thymidine methyl signal upfield peak in Figure 3A was used as a standard for both intensity and chemical shifts (12.9 ppm relative to dioxane at 67.86 ppm).

Table I: ^{13}C Chemical Shifts of Aqueous Solution of Chromomycin A_3 at pH 8.3[a]

peak	ppm[b]	peak	ppm[b]	peak	ppm[b]
1	195	18	80.35	35	43.10
2	175.07	19	78.60	36	37.9
3	174.78	20	75.94	37	36.62
4	162.9	21	74.97	38	33.59
5	159.1	22	73.08	39	32.69
6	139.2	23	72.55	40	28.55
7	138.5	24	71.80	41	22.71
8	113.4	25	71.56	42	22.04
9	112.2	26	70.88	43	21.61
10	111.3	27	69.63	44	21.44
11	102.8	28	69.06	45	19.71
12	100.3	29	68.42	46	18.44
13	99.5	30	66.77	47	18.15
14	98.5	31	66.55	48	17.23
15	96.36	32	62.68	49	17.02
16	94.5	33	59.77	50	8.79 (1)
17	82.01	34	43.73		

[a] Peak 50 was used as a reference (indirectly to dioxane at 67.86 ppm); for other spectral conditions see caption of Figure 1. [b] The number of digits represents the estimated accuracy of the chemical shift values.

Table II: ^{13}C Chemical Shifts and Assignment for the Base Pair Resonances of Calf Thymus DNA Fragments in the Presence of Chromomycin A_3 (CHR)[a]

peak	assignment	pH 8	pH 8 + CHR	pH 5.5 + CHR
1	T4, C4	167.1	167.2	167.0
2	G6	159.8	159.9	159.8
3	C2	158.1	158.3	*b*
4	A6	156.3	156.4	156.3
5	G2	155.2	154.8	154.8
6	A2	153.8	153.8	153.4
7	T2	152.6	152.4	152.3
8	G4	*b*	*b*	*b*
9	A4	149.3	149.3	149.4
10	C6	142.3	142.3	142.6
11	A8	140.6	140.4	140.4
12	G8, T6	138.1	138.3	138.2

[a] Peak numbering as displayed in Figure 2. Other conditions are given in Figure 3. Assignments based on spectra of a mixture of nucleotides (see supplementary material) and previously reported chemical shifts of natural DNA (Bolton & James, 1980; Rill et al., 1980; Levy et al., 1981), biosynthetic polymers (Alderfer & Ts'o, 1977; Shindo, 1981), and monomers (Alderfer et al., 1982; Petersen et al., 1982). [b] An unresolved shoulder peak.

changes as well as some chemical shift changes. Also, it is evident that the chromomycin A_3 molecule is tightly bound to the DNA fragments since the line widths observed for the various methyl resonances of chromomycin are similar to that of the methyl group of thymine (Berman & Shafer, 1983). The ratio of chromomycin to DNA was kept very low in order to ensure complete binding of the drug molecules to DNA. The degree of overlap between the chromomycin carbon resonances and those of DNA was found to be very small in general. Thus, both intensity and amplitude changes between the spectra displayed in Figure 2A,B could be compared directly. Due to resonance overlap, and large initial intensity differences in the region corresponding to peaks 3–9, we have concentrated on the remaining resonances for the analysis of intensity changes. We have found that peaks 1 and 2 were reduced by ~15%. The most noticeable changes were observed for peaks 10–12. Peaks 10 and 12 had reduced intensities of ~45 and ~25%, respectively, while peak 11 showed only a slight intensity decrease (~10%). The smaller intensity decrease for peak 12 is a result of a small contribution from a chromomycin aromatic resonance (Berman & Shafer, 1983). Since peak 1 contains one component of cytidine (C4), peak 2 corresponds to G6, peak 10 corresponds to C6, and one component of peak 12 is G8 (see Table II), it can be concluded that the intensity changes occur predominantly in the G-C base pair resonances in accord with previously reported observations of G-C specificity in the binding of chromomycin (Behr et al., 1969; Van Dyke & Dervan, 1983). The solution conditions and drug/DNA ratio employed in this work probably correspond to interaction of the drug with only one A-T base pair for every four or five G-C base pairs (Van Dyke & Dervan, 1983). Thus, the observed intensity effects on the resonances corresponding to G-C carbons are expected to be more pronounced than those observed for the A-T carbons.

(*2*) *Comparison with Actinomycin–DNA and Hoechst 33258–DNA Complexes.* The spectrum of the chromomycin–DNA sample was compared with the spectrum of an actinomycin–DNA sample under identical conditions (Figure 2B vs. Figure 2C). Within the limits imposed by overlap of the actinomycin resonances and the DNA resonances, it is clear that the same G-C base pair carbon resonances are affected but to a greater degree. On the other hand, the carbon resonances of the polypeptide side chains of actinomycin (structure **2**) seem to have narrower line widths than the corresponding sugar side-chain carbon resonances of chromomycin A_3 (structure **1**). The highly reduced mobility of the sugar residues of chromomycin A_3 is consistent with strong binding to DNA and the observation that the sugar sidechains are needed for binding to DNA (Koshel et al., 1966). But it is surprising that their motion appears to be more restricted than that of the polypeptide side chains of actinomycin D (Berman & Shafer, 1983) which also are believed to be rigid and are bound in the minor groove of DNA (Sobell & Jain, 1972; Brown et al., 1984). When the spectrum of the chromomycin–DNA sample was recorded at two different temperatures (40 vs. 30 °C), the relative increase in the line widths for the chromomycin methyl resonances (90%) was similar to that observed for the T(me) resonance (110%). This provides additional evidence that the sugar side chains of the bound drug are constrained to move with the DNA.

We also obtained the ^{13}C NMR spectrum, under similar conditions of the DNA sample, with Hoechst 33258 which is known to be a nonintercalator with A-T base pair specificity (Latt & Wohlleb, 1975; Muller & Gautier, 1975). The resultant ^{13}C NMR spectrum (not shown) showed smaller spectral intensity changes than those observed for chromomycin, and these were observed principally for the A-T base pair carbon resonances. Thus, intercalating drugs (e.g., actinomycin D) and nonintercalating (e.g., Hoechst 33258) drugs both affect the relative intensity of the base pair carbon resonances but with a marked degree of difference in the magnitude of the observed changes.

(*3*) *pH Effects.* We investigated the pH effect on binding chromomycin to DNA. Since chromomycin has a pK_a of ~7.0, it was of interest to compare the effect of negative charge on binding (the presence of stoichiometric amounts of Mg^{2+} decreased the pK_a of the drug by only 0.2 pK unit). The requirement for Mg^{2+} cations for effective binding of chromomycin to DNA has been interpreted as mediating the negatively charged DNA phosphate groups and the negatively charged chromomycin (Nayak et al., 1973). Thus, the formation of a given chromomycin–DNA complex may ultimately depend on the presence of a negative charge on the drug molecule. For this purpose we have recorded the ^{13}C NMR spectra of chromomycin–DNA sample at different pH values, above and below the pK_a value of chromomycin, as shown in Figure 3. Only a few small chemical shift changes, brought about by the lowering of the pH (see Table II), are observed

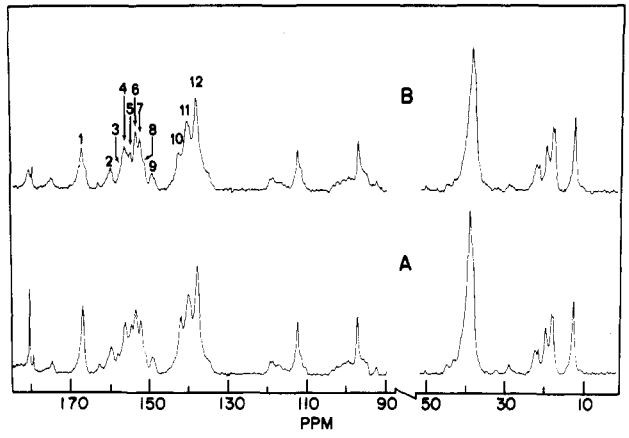


FIGURE 3: 60.46-MHz ^{13}C NMR spectra, displaying the aromatic and upfield region, of 8 mM chromomycin A_3 and 150 mM DNA mixtures at two pH values: (A) pH 8.0, other conditions as for Figure 2B. (B) Same as (A) but at pH 5.5, 37 °C, and 65000 scans.

in the downfield region of the spectrum in analogy with the chemical shift changes observed for the mixture of monomers at similar pH values [data available in supplementary material (see paragraph at end of paper regarding supplementary material)]. The intensity changes for the DNA base resonances seem to be very similar but somewhat more pronounced at the lower pH value. This is particularly evident for peaks 1, 2, 10, and 12. On the other hand, there is little change in the appearance of the bound chromomycin resonances in the spectrum, which is clearly indicated in the upfield region of the spectrum. Thus, it appears that there is no profound change in the structure of the resultant chromomycin–DNA complex with "neutralization" of the negative charge of chromomycin. From the relative intensity changes for the DNA signals it may be inferred that the binding is stronger at the lower pH value, with very little change in the overall structure of the complex.

The fact that the line widths for the chromomycin methyl groups are not broader at the lower pH value is a strong indication of total binding of chromomycin to DNA at both pH values. This may be contrasted with a marked line broadening observed for the aqueous solutions of chromomycin alone at pH 7.3 (Figure 1).

(*4*) *Mg^{2+} Contributions.* Analysis of the ^{13}C NMR results presented in the previous sections indicates that the chromomycin–DNA complex is more tightly bound at solution pH values below the pK_a of chromomycin. This may not seem to be very surprising if the role of the Mg^{2+} cation is to counteract electrostatic repulsions between negatively charged phosphate groups of DNA and anionic chromomycin (Nayak et al., 1973). Since, at lower pH, the drug is a predominantly neutral molecule like actinomycin (structure 2), it may be involved in a tighter binding complex to DNA. In order to investigate this point, we have compared the visible spectrum of chromomycin in the presence of DNA at two pH values with and without Mg^{2+} cations (Figure 4). In all cases we have essentially the same DNA and chromomycin concentrations (1.9 and 0.1 mM, respectively), and where indicated, 0.1 mM Mg^{2+} was added. It is clear from Figure 4 that in the absence of Mg^{2+} traces 2 and 4 both have approximately the same λ_{max} (~400 nm). The difference in shape and extinction coefficient is attributed to the fact that the chromophore is negatively charged at the higher pH value. Furthermore, traces 2 and 4 have the same λ_{max} as found in the absence of DNA (Nayak et al., 1973). Therefore, it appears that either no binding occurs in the absence of Mg^{2+} at both pHs or that complex

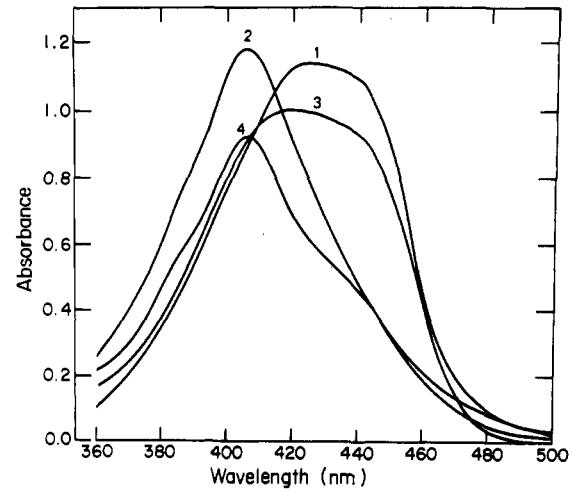


FIGURE 4: Absorbance spectra of equilibrated chromomycin A_3–DNA solutions in the range 360–500 nm. Each solution contained ~1.9 mM DNA and 0.1 mM chromomycin A_3 in 20 mM Tris buffer. Trace 1, in the presence of 0.1 mM $MgCl_2$ at pH 8.4; trace 2, same as trace 1 but no $MgCl_2$; trace 3, in the presence of 0.1 mM $MgCl_2$ at pH 5.5; trace 4, same as trace 3 but no $MgCl_2$.

formation occurs without a shift in λ_{max}. The latter alternative is supported by work of Kersten et al. (1966) showing cosedimentation of DNA and chromomycin in the absence of divalent cations. Addition of stoichiometric amounts of Mg^{2+} shifts the chromophore's spectrum to higher wavelengths for both the high and low pH values (traces 1 and 3, respectively). The general shape of the spectra is very similar, and only a small difference is recorded for their λ_{max} (=424 nm for 1 and 420 nm for 3). The shift to higher wavelength is indicative of strong binding to DNA. The requirement for Mg^{2+} at low pH, where the drug is uncharged, clearly indicates that Mg^{2+} cations are involved in the chromomycin–DNA complex beyond their proposed role of counteracting charges.

It may be that the difference in the observed absorbance ratio at λ_{max} for trace 2/trace 1 (1.04) and trace 4/trace 3 (0.91) reflects a change in the internal pH at the immediate environment of the free drug chromophore. This change may be brought about by the addition of Mg^{2+} and may be attributed to the Mg^{2+} cation promoting deprotonation of the chromophore and thus facilitating the onset of chromomycin–DNA complex formation. An alternative explanation could be that the exctinction coefficients of traces 1 and 3 reflect a decrease and an increase, respectively, in the apparent internal pH values as experienced by the chromophore upon interaction with the DNA. We note that, at elevated Mg^{2+} concentrations (Mg^{2+}/chromomycin > 400), the ratio of absorbance at λ_{max} for bound relative to free drug (Nayak et al., 1973) is reversed from that at stoichiometric levels of Mg^{2+} (~0.91 vs. 1.04).

Low-Field ^1H NMR Spectra

In order to address further the question of binding mode for chromomycin A_3, we examined the imino proton spectra of complexes formed with calf thymus DNA as well as the synthetic alternating polynucleotide poly(dG-dC). Earlier studies by Kearns and co-workers (Feigon et al., 1984) demonstrated the usefulness of this approach to determining base pair specificity as well as distinguishing intercalation from groove binding. Upfield shifts for the imino proton resonances are expected upon binding an intercalator, while downfield shifts typically occur for minor groove binding ligands.

The effect of chromomycin A_3 on the imino protons of calf thymus DNA is shown in Figure 5. The major effect, in addition to a general broadening, on the DNA protons is a

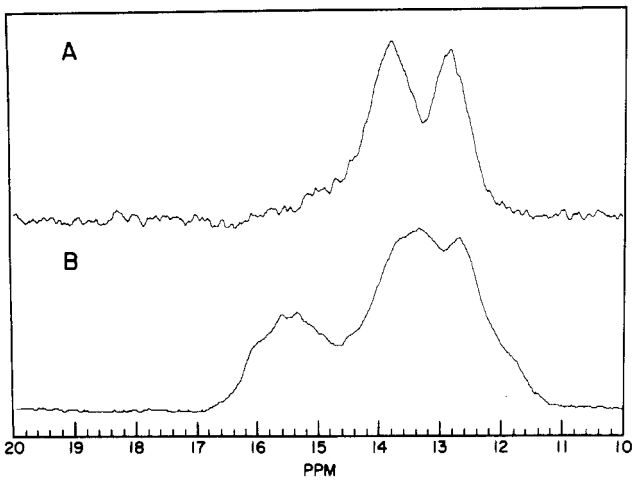


FIGURE 5: Low-field ^1H NMR spectrum of calf thymus DNA fragments (30 mM nucleotide) and Mg^{2+} (9 mM) in the absence (A) and presence (B) of chromomycin A_3 (7 mM). Buffer contained 17 mM Tris, 170 mM NaCl, 0.17 mM EGTA, and 10% D_2O, pH 7.4, 15 °C. Spectra are plotted with 15-Hz line broadening and were obtained in 256 (A) or 9216 scans (B).

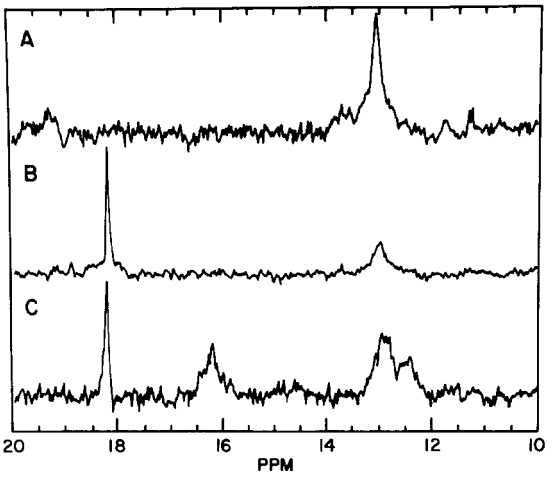


FIGURE 6: Effect of chromomycin and Mg^{2+} on low-field ^1H NMR spectrum of sonicated poly(dG-dC) in 50 mM sodium phosphate, 200 mM NaCl, 0.1 mM EGTA, and 10% D_2O, pH 7.4, 15 °C. (A) poly(dG-dC) (8 mM nucleotide); (B) poly(dG-dC) (8 mM nucleotide) + chromomycin A_3 (4 mM); (C) poly(dG-dC) (32 mM nucleotide) + $MgCl_2$ (4 mM) + chromomycin A_3 (4 mM). Spectra are plotted with 3-Hz line broadening and were obtained in 2000–4000 scans.

small shoulder on the upfield side of the G-C peak. As mentioned earlier, it is well established that chromomycin has a strong preference for G-C sites (Behr et al., 1969). Hence, the observed upfield shift is consistent with an intercalative type of binding. In fact, the effect of chromomycin on the G-C resonance in Figure 5 is similar to that reported by Feigon et al. (1984) for actinomycin.

The most striking aspect of Figure 5 is a new, broad peak centered at 15.5 ppm in the drug complex not found with DNA alone. This resonance most likely arises from the phenolic hydroxyl proton attached to the central ring of the chromophore. The large line width of this peak indicates that it arises from a DNA-bound form of the drug. A peak around 15 ppm was reported by Miyamoto et al. (1964) for a derivative of chromomycin in $CDCl_3$ and was assigned to the phenolic hydroxyl proton that can form a hydrogen bond with the adjacent carbonyl oxygen. As the pH of our samples for the proton NMR study was close to the pK_a of the drug, this peak should be observed, provided its exchange with H_2O is sufficiently slow.

It is of interest to note that the A-T imino peak is also shifted upfield in the presence of chromomycin. Thus, while there is a G-C preference for binding chromomycin, A-T base pairs are affected as well. This is consistent with the footprinting study of Van Dyke & Dervan (1983) which showed that the two strongest binding sites for this drug on a DNA restriction fragment were 3′-GGG and 3′-CGA.

The very broad resonances seen in Figure 5 are due in large part to the heterogeneity of natural DNA. Considerably sharper lines can be seen in Figure 6 where sonicated poly(dG-dC) was used. The spectrum in Figure 6A is that of poly(dG-dC) alone, showing the imino proton resonance near 13 ppm. Figure 6B displays the spectrum of poly(dG-dC) in the presence of chromomycin A_3 but without any Mg^{2+}. In this spectrum, the G-C imino proton resonance at 13 ppm is similar to that in Figure 6A, but there is a new, sharp peak near 18 ppm. This is attributed to the 9-OH proton of the drug either in the unbound form or in a weakly bound form characteristic of conditions lacking in divalent cations. As mentioned above, Kersten et al. (1966) have demonstrated that chromomycin A_3 can interact with DNA in the absence of divalent cations. Thus, it is possible that the sharp peak in Figure 6B is due to such a bound form. The relatively narrow line width of this peak suggests that the drug has considerable mobility in such a complex.

In Figure 6C, where both chromomycin and Mg^{2+} have been added to poly(dG-dC), two additional peaks appear. There is a new peak upfield from the G-C imino resonance, also seen in Figure 5B, although here it is better resolved due to the narrower line widths in general. This new upfield peak is again consistent with the notion that the drug binds via intercalation. The second new peak occurs near 16 ppm and is similar in line width to that of the imino proton peak. This peak is attributed to the 9-OH proton of chromomycin in the "tight" complex with DNA, i.e., in the complex formed in the presence of divalent cations. The presence of the relatively sharp peak at 18 ppm indicates that both forms of the bound drug (or both bound and free drug) are present and in slow exchange. Furthermore, the position of the broad resonance from the 9-OH proton in the "tight" complex is upfield from the narrow peak near 18 ppm. This upfield shift is again expected for binding by intercalation.

It is of interest to compare Figure 6C with Figure 5B, where there is no sharp peak downfield from that of 15.5 ppm. It appears that not all the drug is bound in the tight complex in the case of poly(dG-dC). This may be due to the fact that the concentration of Mg^{2+} is greater in the calf thymus sample and/or that there are more binding sites on calf thymus DNA than on poly(dG-dC). Evidence for the latter comes from the Van Dyke and Dervan study (1983) in which all the chromomycin binding sites identified on a restriction fragment consisted of sequences of two or three purines on one strand. Such a sequence is clearly not available on the alternating synthetic polynucleotide.

CONCLUSIONS

The results of our ^1H NMR experiments provide evidence that, in the presence of Mg^{2+}, chromomycin A_3 binds to DNA by intercalation. In particular, the upfield shifts in both the calf thymus DNA and poly(dG-dC) imino protons in the drug complex are consistent with other studies on the effects of intercalators on DNA imino protons (Feigon et al., 1984). In contrast, the bulk of the previously reported data on the binding of chromomycin A_3 to DNA appears contrary to the suggestion of an intercalative mode of binding. However, the experimental conditions were often not those that favored the

"tight" binding which produces the shift in the visible spectrum of the bound drug to longer wavelengths as well as the upfield shifts in the imino proton region of the NMR spectrum. Thus, Kersten et al. (1966) reported that chromomycin did not affect the thermal denaturation of DNA and did not alter the sedimentation or viscosity of linear DNA. But these experiments were all carried out in the absence of Mg^{2+} or other divalent cations.

One of the most important pieces of evidence pertinent to the question of binding modes comes from the unwinding study of Waring (1970). In that work, it was shown that chromomycin A_3 produced a small decrease in the sedimentation coefficient of supercoiled DNA but never yielded a minimum with a subsequent increase with increasing drug levels. In this case, relatively low levels of Mg^{2+} were used, i.e., 0.1 mM, and at the highest drug concentration, it was estimated that only 13% of the drug was bound to the DNA. It is possible that at this concentration of Mg^{2+}, a significant proportion of the drug was bound in the "weak" mode and that under conditions of higher divalent cation concentration more of the drug could bind by the "strong" mode. Such conditions may indeed lead to the minimum typical for intercalators in a plot of sedimentation coefficient of supercoiled DNA as a function of drug concentration.

The ^{13}C NMR spectra presented for the chromomycin A_3–DNA sample clearly demonstrate the G-C base pair specificity of the drug, as the major spectral changes occur for signals assigned to G-C base pair carbons. The similarity in the general kinetic and thermodynamic properties, the base pair specificity, and the ^{13}C NMR spectral changes observed for chromomycin–DNA and actinomycin–DNA complexes suggest that their mode of binding may be very similar. At lower pH the similarity to actinomycin–DNA complex is even greater. These results also lend support to the notion of intercalative binding for chromomycin. The precise role of Mg^{2+} remains uncertain, although the results presented here indicate that it may go beyond more neutralization of the anionic form of the drug. More detailed studies using two-dimensional techniques on oligonucleotide complexes are under way and should provide a more definitive picture of the drug–DNA structure.

We have also observed that the sugar residues are tightly bound in the DNA complex which directly confirms the conclusion that the sugar residues are important for effective binding of the drug to DNA. They are in fact probably bound to DNA more rigidly than the polypeptide side chains of actinomycin D. The role played by the sugar residues of chromomycin may be duplicated by the sugar residues of other antibiotics which bind to DNA such as bleomycin or daunomycin.

Added in Proof

Preliminary experiments on an oligonucleotide–chromomycin complex show effects in the imino proton region similar to those presented above, but NOE measurements suggest a nonintercalative binding interaction.

Supplementary Material Available

A figure comparing the ^{13}C NMR spectrum of calf thymus DNA with that of a mixture of the four nucleotides and a table listing chemical shifts (2 pages). Ordering information is given on any current masthead page.

Registry No. Mg, 7439-95-4.

References

Alderfer, J. L., & Ts'o, P. O. P. (1977) *Biochemistry 16*, 2410–2416.

Alderfer, J. L., Loomis, R. L., & Zielinski, T. J. (1982) *Biochemistry 21*, 2738–2745.

Behr, W., Honikel, K., & Hartmann, G. (1969) *Eur. J. Biochem. 9*, 82–92.

Berman, E., & Shafer, R. H. (1983) *Biopolymers 22*, 2163–2167.

Bolton, P., & James, T. (1980) *Biochemistry 19*, 1388–1392.

Brown, S. C., Shafer, R. H., & Mirau, P. A. (1982) *J. Am. Chem. Soc. 104*, 5504–5506.

Brown, S. C., Mullis K., Levenson, C., & Shafer, R. H. (1984) *Biochemistry 23*, 403–408.

Feigon, J., Denny, W. A., Leupin, W., & Kearns, D. R. (1984) *J. Med. Chem. 27*, 450–465.

Hayasaka, T., & Inoue, Y. (1969) *Biochemistry 8*, 2342–2347.

Kersten, W., Kersten, H., & Szybalski, W. (1966) *Biochemistry 5*, 236–244.

Koshel, K., Hartmann, G., Kersten, W., & Kersten, H. (1966) *Biochem. Z. 344*, 76–86.

Krugh, T. R., & Chen, Y.-C. (1975) *Biochemistry 14*, 4912–4922.

Krugh, T. R., & Chen Chiao, Y.-C. (1977) *Biochemistry 16*, 747–755.

Latt, S. A., & Wohlleb, J. C. (1975) *Chromosoma 52*, 297–316.

Levy, G. C., Hilliard, P. R., Jr., Levy, L. F., & Rill, R. L. (1981) *J. Biol. Chem. 265*, 9986–9989.

Miyamoto, M., Morita, K., Kawamatsu, Y., Noguchi, S., Marumoto, R., Tanaka, K., Tatsuoko, S., Nakanishi, K., Nakadaira, Y., & Bhacca, N. S. (1964) *Tetrahedron Lett.* 2355–2365.

Miyamoto, M., Kawamatsu, Y., Kawashima, K., Shinohara, M., Tanaka, K., Tatsuoka, S., & Nakanishi, K. (1967) *Tetrahedron 23*, 421–437.

Muller, W., & Gautier, F. (1975) *Eur. J. Biochem. 54*, 385–394.

Nayak, R., Sirsi, M., & Podder, S. K. (1973) *FEBS Lett. 30*, 157–162.

Pardi, A., Morden, K. M., Patel, D. J., & Tinoco, I., Jr. (1983) *Biochemistry 22*, 1107–1113.

Patel, D. J. (1974a) *Biochemistry 13*, 1476–1482.

Patel, D. J. (1974b) *Biochemistry 13*, 2388–2395.

Patel, D. J. (1974c) *Biochemistry 13*, 2396–2402.

Petersen, S. B., Led, J. J., Johnston, E. R., & Grant, D. M. (1982) *J. Am. Chem. Soc. 104*, 5007–5015.

Plateau, P., & Gueron, M. (1982) *J. Am. Chem. Soc. 104*, 7310–7311.

Remers, W. A. (1979) in *The Chemistry of Antitumor Antibiotics*, Vol. 1, pp 133–175, Wiley, New York.

Rill, R. L., Hilliard, P. R., Jr., Bailey, J. T., & Levy G. C. (1980) *J. Am. Chem. Soc. 102*, 418–420.

Shapiro, H. S. (1968) *Handbook of Biochemistry—Selected Data for Molecular Biology* (Sober, H. A., Ed.) H-30, CRC Press, Cleveland, OH.

Shindo, H. (1981) *Eur. J. Biochem. 120*, 309–312.

Thiem, Y., & Meyer, B. (1979) *J. Chem. Soc., Perkin Trans. 2*, 1331–1336.

Thiem, T., & Meyer, B. (1981) *Tetrahedron 37*, 551–558.

Van Dyke, M. W., & Dervan, P. B. (1983) *Biochemistry 22*, 2372–2377.

Waring, M. J. (1970) *J. Mol. Biol. 54*, 247–280.